



Consommation et Corporations Canada Consumer and Corporate Affairs Canada

Bureau des brevets Patent Office

Ontario Canada  
M1A 0C9

(21) (A1) 2,062,047  
(22) 1992/02/28  
(43) 1992/08/29

(51) INTL. CL. <sup>5</sup> C12P-021/08; C12N-015/62; C12N-005/10

(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Fusion Proteins for Prodrug Activation, the Preparation and Use Thereof

(72) Seemann, Gerhard - Germany (Federal Republic of) ;  
Bosslet, Klaus - Germany (Federal Republic of) ;  
Czech, Joerg - Germany (Federal Republic of) ;  
Kolar, Cenek - Germany (Federal Republic of) ;  
Hoffman, Dieter - Germany (Federal Republic of) ;  
Sedlacek, Hans-Harald - Germany (Federal Republic of) ;

(73) Behringwerke Aktiengesellschaft - Germany (Federal Republic of) ;

(20) (DE) P 41 06 389.9 1991/02/28

(57) 13 Claims

Notice: The specification contained herein as filed

Canada

## Abstract of the Disclosure

Fusion proteins for prodrug activation, the preparation and use thereof

---

The invention relates to fusion proteins for prodrug activation of the general formula huTumAb-L- $\beta$ -Gluc, where huTumAb is a humanized or human tumor-specific monoclonal antibody or a fragment thereof, L is linker, and  $\beta$ -Gluc comprises human  $\beta$ -glucuronidase. These fusion proteins are prepared by genetic manipulation. huTumAb ensures the specific localization of tumors, L connects huTumAb to  $\beta$ -Gluc in such a way that the specific properties of the two fusion partners are not impaired, and  $\beta$ -Gluc activates a suitable prodrug compound by elimination of glucuronic acid, where a virtually autologous system for use in humans is provided by the humanized or human fusion partners.

2062017

BEHRINGWATTE ARTFABRIKSGESellschaft

MOE 91/B 004 - Ma 276

Dr. Lp/Bi

Description.

- 5 Fusion proteins for prodrug activation, the preparation and use thereof

10 The invention relates to fusion proteins for prodrug activation of the general formula huTuMab-L- $\beta$ -Gluc, where huTuMab is a humanized or human tumor-specific monoclonal antibody, a fragment or a derivative thereof, L is linker, and  $\beta$ -Gluc comprises human  $\beta$ -glucuronidase. These fusion proteins are prepared by genetic manipulation. huTuMab ensures the specific localization of tumors, L connects huTuMab to  $\beta$ -Gluc in such a way that the specific properties of the two fusion partners are not impaired, and  $\beta$ -Gluc activates a suitable prodrug compound by elimination of glucuronic acid, where a virtually autologous system for use in humans is provided  
15 by the humanized or human fusion partners.  
20

25 Combination of prodrug and tumor-specific antibody-enzyme conjugates for use as therapeutic agents is described in the specialist literature. This has entailed injection of antibodies which are directed against particular tissue and to which a prodrug-cleaving enzyme is covalently bonded, into an animal which contains the transplanted tissue, and subsequently administering a prodrug compound which can be activated by the enzyme. The action of the antibody-enzyme conjugate which is anchored in the tissue  
30 converts the prodrug compound into the cytotoxin which exerts a cytotoxic effect on the transplanted tissue.

35 WO 88/07378 describes a therapeutic system which contains two components and is composed of an antibody-enzyme component. The use of non-mammalian enzymes for the preparation of the antibody-enzyme conjugate is described in this case, and that of endogenous enzymes is ruled out

because of the non-specific release of active substance. Since the exogenous enzymes are recognized as foreign antigens by the body, the use thereof is associated with the disadvantage of an immune response to these non-  
5 endogenous substances, on the basis of which the enzyme immobilized on the antibody is inactivated and, possibly, the entire conjugate is eliminated. Furthermore, in this case p-bis-M-(2-chloroethyl)-amino-benzylglutamic acid and derivatives thereof are used as prodrug, the chemical  
10 half-life thereof being only 5.3 to 16.5 hours. The chemical instability of a prodrug compound is a disadvantage because of the side effects to be expected.

EP A2-0 302 473 likewise describes a therapeutic system containing two components, in which the antibody-enzyme  
15 conjugate which is localized on the tumor tissue cleaves a prodrug compound to give a cytotoxic active substance. The combined use, which is described herein inter alia, of etoposide 4'-phosphate and derivatives thereof as  
20 prodrug and antibody-immobilized alkaline phosphatases to liberate the etoposides is a disadvantage because of the presence of large amounts of endogenous alkaline phosphatases in serum. DE A1-38 26 562 describes how etoposide  
25 4'-phosphates have already been used alone as therapeutic antitumor agent, with the phosphatases present in serum liberating the etoposide from the prodrug.

It has been found that huTUMAbs coupled via L to  $\beta$ -Gluc and prepared by genetic manipulation represent a  
30 particularly advantageous, because virtually autologous, system. It has additionally been found that the catalytic activity of  $\beta$ -Gluc in the fusion protein at pH 7.4 (i.e. physiological conditions) is significantly higher than  
35 that of the native enzyme when the fusion protein is bound to the antigen via the V region. Furthermore, a fusion protein with only one hinge region (see Fig. P and Example O) can be generated by genetic manipulation in high yield because most of the product which is formed

2052017

results as one band (in this case with molecular weight 125,000) and can easily be purified by affinity chromatography with anti-idiotypic MAbs or anti-glucuronidase MAbs.

5 It has furthermore been found that a chemical modification of the fusion proteins, in particular partial or complete oxidation of the carbohydrate structures, preferably with subsequent reductive amination, results in an increased half-life. Enzymatic  
10 treatment of the fusion proteins according to the invention with alkaline phosphatase from, for example, bovine intestine or E. coli has in general not resulted in a significant increase in the half-life.

15 Consequently, the invention relates to fusion proteins of the formula

huTuMAB-L- $\beta$ -Gluc (I)

where huTuMAB is a humanized or human tumor-specific  
monoclonal antibody or a fragment or a derivative  
thereof, and preferably comprises the MAbs  
20 described in EP-A1-0 388 914. The fusion proteins according to the invention particularly preferably contain the humanized MAB fragment with the V<sub>H</sub> and V<sub>L</sub> genes shown in Table 3.

25 L is a linker and preferably contains a hinge region of an immunoglobulin which is linked via a peptide sequence to the N-terminus of the mature enzyme.

$\beta$ -Gluc is the complete amino-acid sequence of human  $\beta$ -glucuronidase or, in the relevant gene constructs, the complete cDNA (Oshima A. et al.,  
30 Proc. Natl. Acad. Sci. USA 84, (1987) 685-689.

Furthermore preferred are constructs with a CH<sub>1</sub> exon and a hinge exon in the antibody part, and particularly preferred constructs are those in which these parts

*Handwritten:*  
Dated  
2000/01/17  
Lust

derive from a human IgG3 C gene. Most preferred are constructs, as described in Example (I), where the corresponding light chain of the humanized TuMAb is co-expressed in order, in this way, to obtain an huTuMAb portion which is as similar as possible to the original TuMAb in the binding properties. Finally, the invention relates to processes for the preparation by genetic manipulation of the abovementioned fusion proteins, to the purification thereof and to the use thereof as pharmaceuticals. Fusion proteins as described can be used for prodrug activation in oncoses.

In another embodiment, the fusion proteins according to the invention are chemically modified in order to achieve an increased half-life and thus an improved localization of tumors. The fusion proteins are preferably treated with an oxidizing agent, for example periodate, which generally results in partial or complete cleavage of the carbohydrate rings and thus in an alteration in the carbohydrate structure. This alteration generally results in an increased half-life. It is furthermore advantageous to derivatize, in a second reaction step, existing aldehyde groups, for example by reductive amination. The partial or complete destruction of the aldehyde groups generally results in a reduction in possible side reactions with, for example, plasma proteins. Accordingly, it is advantageous for the fusion proteins according to the invention to be oxidized in a first reaction step, for example with periodate, and to be reductively aminated in a second reaction step, for example with ethanolamine and cyanoborohydride.

The following examples describe the synthesis by genetic manipulation of a particularly preferred fusion protein according to the invention, the derivatization thereof and the demonstration of the ability of the two fusion partners to function.

**Example (A):**

5 The starting material was the plasmid pGEM4-HUGP13 (Fig. A). pGEM4-HUGP13 contains a cDNA insert which contains the complete coding sequence for the human  $\beta$ -glucuronidase enzyme (Oshima et al. loc. cit.). All the techniques used were taken from Maniatis et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor USA (1982).

10 **Example (B):**

The plasmid pGEM4-HUGP13 was cut with the restriction endonucleases PstI and SalI, and the 342bp-long PstI/SalI fragment which harbors the NotI restriction cleavage site was isolated. The PstI/SalI fragment was cloned into the 15 PstI/SalI-cleaved vector pTZ (Fig. B.1) and the clone pTZ;Glc350 was isolated (Fig. B.2).

**Example (C):**

20 The plasmid clone pTZ;Glc350 was cleaved with PstI, and the double-stranded DNA fragment  $\beta$ Glc linker, which is composed of the oligonucleotides  $\beta$ Glc linker 1 and  $\beta$ Glc linker 2 (Tab. 1) and has cohesive PstI ends, was ligated into the opened PstI cleavage site.

Tab. 1

2Glc 370-1

5' GGG GGG GGG GGG GTC CA 3'

2Glc 370-2

5' GGG GGG GGG GGG GTC CA 3'

5 The clone pTZ $\beta$ Glc370 in which the  $\beta$ -glucuronidase frag-  
ment is extended at its 5' end by oligonucleotide 1 and  
which has lost the previously present PstI cleavage site  
but has acquired instead at its 5' end a new PstI  
cleavage site was isolated (Fig. C).

Example (D):

10 The plasmid clone pTZ $\beta$ Glc370 was cleaved with PstI and  
ligated to the hinge-linker fragment which is composed of  
the hinge oligonucleotides 1 and 2b and which has two  
cohesive PstI ends (Tab. 2). This results in the PstI  
cleavage site at the 5' end of the  $\beta$ Glc370 fragment being  
destroyed. The plasmid clone pTZ $\beta$ Glc420 in which the  $\beta$ Glc  
15 insert is extended at the 5' end by the hinge linker R  
was isolated (Fig. D).



Tab. 2

Hinge 1 - CH<sub>1</sub> Cys

5' GAG CCC AAA TGT TGT GAG ACA CCG CCC GCG TGC CCA  
CGG TGC CCA GTT GCA

Hinge 2 - CH<sub>1</sub> Cys

5' ACT GCG CAC CCG GCG CAC GCG GCA CCG GTC TCA CAA  
GAT TTG GCG TGT GCA

Example (E):

5 The plasmid pTZ $\beta$ Glc420 was cleaved with PstI and SalI,  
and the 420bp insert was isolated. The plasmid  
IgG3c F(ab')<sub>2</sub> 2H (EP-A2-0 352 761, Fig. 3, ibidem), which  
contains the CH<sub>1</sub> exon and two hinge exons of a human IgG3  
C gene was completely cleaved with SalI and partially  
cleaved with PstI. The isolated 420bp insert was ligated  
10 to this SalI/PstI (part.)-cleaved plasmid, and the  
plasmid clone which contains the CH<sub>1</sub> exon, a hinge exon  
and the  $\beta$ Glc420 fragment, that is to say carries the  
genetic information of two hinge exons between CH<sub>1</sub> exon  
and  $\beta$ -glucuronidase, was isolated (pUC CH<sub>1</sub> + H +  $\beta$ Glc420)  
(Fig. E).

Tab. 3

422/25 VZ hum

10	30	50
caactgcaggagagcggccaggtcttggagacctaagccagacctaagcctgagcctgaccta		
GlnLeuGlnGlnSerGlyProGlyLeuValArgProSerGlnThrLeuSerLeuThrQ		
70	90	110
accgtgtctggcttcacccatcagcagtggttatagctggcactgggtgagacagccac		
ThrValSerGlyPheThrIleSerSerGlyTyrSerTrpHisTrpValArgGlnProP		
130	150	170
ggacgaggtcttgagtggttggatcactcagtcacagtggtatcactaactacaacc		
GlyArgGlyLeuGlnTrpIleGlyTyrIleGlnTyrSerGlyIleThrLeuTyrAsnP		
190	210	230
tctctcaaaagtagagtgacaatgtctggtagacaccagcaagaaccagtcagcccgat		
SerLeuLysSerArgValThrMetLeuValAspTrpSerLysAsnGlnPheSerLeuA		
250	270	290
ctcagcaggtgacagcggcagacaccgggtctattattgtgcagagagactatgt		
LeuSerSerValThrAlaAlaAspThrAlaValTyrTyrCysAlaArgGluAspTyrA		
310	330	350
taccactggtactctcgatgtctgggtcgaagtcaggtctcgtcacagtctctca		
TyrHisTrpTyrPheAspValTrpGlyGlnGlyTyrLeuValThrValSerSer		

422/25 VZ hum

10	30	50
ggtgtccactccgacatccagatgacccagagcccaagcagcctgagcggcagcgtggt		
GlyValHisSerAspIleGlnMetThrGlnSerProSerSerLeuSerAlaSerValG		
70	90	110
gacagagtgcacatccactgtagtagcagcttcagagtgtaagttacatgcactggtacc		
AspArgValThrIleThrCysSerThrSerSerSerValSerTyrMetHisTrpTyrG		
130	150	170
cagaagccaggttaaggtcccaaaagctgctgatctacagccacatccaaactgggtctctg		
GlnLysProGlyLysAlaProLysLeuLeuIleTyrSerThrSerAsnLeuAlaSerG		
190	210	230
gtgccaaagcagattcagcgttagcgttagcgttagcgttagcgttagcgttagcgttagc		
ValProSerArgPheSerGlySerGlySerGlyThrAspPheThrPheThrIleSerS		
250	270	290
ctccagccagaggtacatccgacactactactgccatcagtgagtagttatcccagct		
LeuGlnProGluAspIleAlaThrTyrTyrCysHisGlnTrpSerSerTyrProThrP		
310	330	
gtgccaaaggtgacaaaggtggaatccaaacgt		
GlyGlnGlyThrLysValGluIleLysArg		

FIG. 10 (F)

The plasmid pGEM4-HUGP13 $\beta$ Glc was cleaved with SalI, and the 1750bp SalI fragment from the  $\beta$ -glucuronidase cDNA was isolated. The isolated 1750bp SalI fragment was ligated to the SalI-cleaved plasmid pUC CH<sub>1</sub> + H +  $\beta$ Glc420. The plasmid clone pUC CH<sub>1</sub> + H + hu $\beta$ Glc which contains a fusion gene composed of a CH<sub>1</sub> exon, a hinge exon and a fusion exon between a hinge exon and the human  $\beta$ -glucuronidase cDNA was isolated (Fig. F).

10 Example (G):

The expression vector pABstop (Fig. I ) was cleaved with HindIII and SalI. The plasmid pUC CH<sub>1</sub> + H + hu $\beta$ Glc was cleaved completely with HindIII and partially with SalI, and the CH<sub>1</sub> + H + hu $\beta$ Glc insert was isolated. The CH<sub>1</sub> + H + hu $\beta$ Glc insert was ligated to the HindIII/SalI-cleaved pABstop, and the clone pABstop CH<sub>1</sub> + H + hu $\beta$ Glc was isolated (Fig. G).

Example (H):

The pABstop vector pABstop BW 431/26 hum V<sub>H</sub>, which contains the humanized version of the V<sub>H</sub> gene of the anti-CEA MAb BW 431/26 (Bosslet K. et al., Eur. J. Nucl. Med. 14, (1988) 523-528) (see Tab. 3 for the sequences of the humanized V<sub>H</sub> and V<sub>L</sub> gene), was cleaved with HindIII and BamHI, and the insert which contains the signal exon and the V<sub>H</sub> exon was isolated. The plasmid clone pABstop CH<sub>1</sub> + H + hu $\beta$ Glc was cleaved with HindIII and ligated to the HindIII/BamHI 431/26 hum V<sub>H</sub> fragment. After ligation at room temperature for 2 h, the ligation was stopped by incubation at 70°C for 10', and the ends which were still free were filled in with Klenow polymerase and dNTPs. Further ligation was then carried out overnight. After transformation, the clone pABstop 431/26 hum V<sub>H</sub> hu $\beta$ Glc1H which contains an immunoglobulin F(ab')<sub>2</sub> gene with a hinge exon, which is fused to the coding region of human

$\beta$ -glucuronidase, was isolated with the aid of restriction mapping and nucleic acid sequence analysis (Fig. H).

Example (I):

5 The clone pABStop 431/26 hum V<sub>H</sub> hu $\beta$ Glc1H was transfected together with a plasmid clone which carries the light chain of humanized BW 431/26 (Fig. I) and two plasmids which carry a neomycin- (Fig. K) and a methotrexate-resistance gene (Fig. L) into BHK cells. A fusion protein which has both the antigen-binding properties of  
10 MAh BW 431/26hum and the enzymatic activity of human  $\beta$ -glucuronidase was expressed.

Example (J):

Demonstration of the antigen-binding properties and of the enzymatic activity of the 431/26 hum V<sub>H</sub>hu $\beta$ Glc 1H fusion protein  
15

The ability of the 431/26 hum V<sub>H</sub>hu $\beta$ Glc 1H fusion protein to bind specifically to the epitope defined by 431/26 on CEA (carcinoembryonic antigen) and, at the same time, to exert the enzymatic activity of human  $\beta$ -glucuronidase was  
20 determined in a specificity/enzyme activity assay. This assay is carried out as described below:

- Polystyrene (96-well) microtiter plates (U shape, Type B, supplied by Nunc, Order No. 4-60445) are incubated with purified CEA (1-5  $\mu$ g of CEA/ml, 75  $\mu$ l of this per well) or with GIT mucin (same amount as CEA) at R.T. overnight.  
25
- The non-adsorbed antigen is removed by aspiration and washed 3 x with 0.05 M tris/citrate buffer, pH 7.4.
- 30 - The microtiter plates are left to stand at R.T. with the opening facing downwards on cellulose overnight.
- The microtiter plates are incubated with 250  $\mu$ l of 1% strength casein solution in PBS, pH 7.2, per well

- (blocking solution) at 20°C for 30 minutes.
- During the blocking, the substrate is made up. The amount of substrate depends on the number of supernatants to be assayed. The substrate is made up fresh for each assay.
  - 5     - Substrate: 4-methylumbelliferyl  $\beta$ -D-glucuronide (Order No.: M-9130 from Sigma), 2.5 mM in 200 mM sodium acetate buffer, pH 5.0, with 0.01% BSA.
  - 10    - The blocking solution is removed by aspiration, and in each case 50  $\mu$ l of BHK cell supernatant which contains the fusion protein are loaded onto the microtiter plate coated with CEA or GIT mucin (that is to say the sample volume required is at least 120  $\mu$ l).
  - 15    - Incubation at R.T. is then carried out for 30 minutes.
  - The plates are washed 3 x with ELISA washing buffer (Behring, OSEW 96).
  - The substrate is loaded in (50  $\mu$ l/well) and incubated at 37°C for 2 hours. The plate is covered because of the possibility of evaporation.
  - 20    - After 2 hours, 150  $\mu$ l of stop solution are pipetted into each well (stop solution = 0.2 M glycine + 0.2% SDS, pH 11.7).
  - 25    - Evaluation can now be carried out under a UV lamp (excitation energy 380 nm) or in a fluorescence measuring instrument (Fluorescan II, ICN Biomedicals, Cat. No.: 78-611-00).

30     It was possible to show using this specificity/enzyme activity assay that fluorescent 4-methylumbelliferol was detectable in the wells coated with CEA when the enzyme activity was determined at pH 5, the catalytic optimum (Tab. 4).

Table 4:

Dilution out of fusion protein cell culture supernatant (R73/2) on CEA and GIT mucin

5	Dilution steps	Substrate in various solutions		
		0.2 M sodium acetate buffer + 0.01% BSA, pH 5, on CEA	PBS, pH 7.2, on CEA	PBS, pH 7.2 on GIT mucin
10	Concen- trated	9118	2725	115.7
	1:2	7678	2141	93.37
	1:4	4662	1195	73.39
	1:8	2927	618.5	60.68
15	1:16	1657	332.1	53.69
	1:32	853	168.2	40.44
	1:64	425	99.26	48.21
	1:128	192.5	57.89	47.48

20 Determination of the conversion rate at pH 7.2 showed that at this physiological pH that of the fusion protein was still ~ 25% of the conversion rate at pH 5. No significant methylumbelliferol liberation was measurable on the negative control plates coated with GIT mucin and measured at pH 5. This finding shows that the humanized

25 V region of the 431/26hum V<sub>h</sub>hu $\beta$ Glc 1H fusion protein has retained its epitope specificity, and the  $\beta$ -glucuronidase portion of the fusion protein is able, like the native human enzyme, to cleave the  $\beta$ -glucuronide of 4-methylumbelliferol.

30 Example (K):

Demonstration of the functional identity of the V region of the 431/26hum V<sub>h</sub>hu $\beta$ Glc 1H fusion protein with that of the humanized MAb BW 431/26 and that of the murine MAb BW 431/26

It was shown in Example (J) that the 431/26<sub>hum</sub> V<sub>h</sub>u<sub>5</sub>Glc 1H fusion protein has a certain CEA-binding potential and  $\beta$ -glucuronidase activity. The antigen-specific competitive assay described hereinafter provides information on the identity of the CEA epitopes which are recognized by the competing molecules, and on the strength of the epitope/fusion protein and epitope/antibody interactions.

This assay is carried out as described below:

- Polystyrene 96-well microtiter plates (U shape, Type B, supplied by Nunc, Order No. 4-60445) are incubated with purified CEA (1-5  $\mu$ g of CEA/ml, 75  $\mu$ l of this per well) or with GIT mucin (same amount as CEA) at R.T. overnight.
- The non-adsorbed antigen is removed by aspiration and washed 3 x with 0.05 M tris/citrate buffer, pH 7.4.
- The microtiter plates are left to stand at R.T. with the opening facing downwards on cellulose overnight.
- The microtiter plates are incubated with 250  $\mu$ l of 1% strength casein solution in PBS, pH 7.2, per well (blocking solution) at R.T. for 30 minutes.
- 50  $\mu$ l of the MAb BW 431/26 in a concentration of 5 ng/ml are mixed with 50  $\mu$ l of 10-fold concentrated supernatant of the humanized MAb BW 431/26 or of the fusion protein, as well as serial 2 x dilutions.
- 50  $\mu$ l aliquots of these mixtures are pipetted into the wells of microtiter plates coated with CEA or GIT mucin.
- The microtiter plates are incubated at R.T. for 30 minutes.
- The plates are then washed 3 x with ELISA washing buffer (supplied by Behringwerke AG, Order No. OSEW 96, 250  $\mu$ l).
- Then 50  $\mu$ l of a 1:250-diluted goat anti-mouse Ig antibody which is coupled to alkaline phosphatase (Southern Biotechnology Associates, Order No.: 1010-04) are added.

- After incubation at R.T. for 30 minutes and washing 3 times, the substrate reaction is carried out as follows:
- Add 30  $\mu$ l of 0.1 mM NADP per well (dissolve 7.65 mg in 100 ml of 20 mM tris; 0.1 mM  $MgSO_4$ , pH 9.5); the solution can be stored at  $-20^\circ C$  for several months.
- Incubate at R.T. for 30 minutes.
- Make up the enhancer system during the incubation with NADP: (5 ml per plate)
- 10 2 parts of INT (dissolve 2.5 mg/ml in 30% strength ethanol in an ultrasonic bath; always make up fresh)  
+ 1 part of PBS, pH 7.2  
+ 1 part of diaphorase (1 mg/ml PBS, pH 7.2)  
+ 1 part of ADH (0.5 mg/ml PBS, pH 7.2)
- 15 - add 50  $\mu$ l of the enhancer system
- when the extent of reaction is as required, stop the reaction with 0.1 N  $H_2SO_4$ , 100  $\mu$ l per well
- measure at 492 nm in a TITERTEK<sup>®</sup> MULTISCAN (blank = 50  $\mu$ l of NADP + 50  $\mu$ l, of enhancer solution +  
20 100  $\mu$ l of 0.1 N  $H_2SO_4$ )

NADP - supplied by Sigma, Order No. N-0505

INT - supplied by Sigma, Order No. I-8377

ADH - supplied by Sigma, Order No. A-3263

Diaphorase - supplied by Sigma, Order No. D-2381

- 25 Reduction of the extinction in this antigen-specific competitive assay means that there is competition between the molecules competing with one another for epitopes which are the same or lying very close together spatially.
- 30 The inhibition data which are obtained show that both the fusion protein 431/26hum V<sub>h</sub>huGlc 1H and the humanized MAb 431/26 block binding of the murine MAb EW 431/26 to its CEA epitope. 50% inhibition is reached at a 200 molar excess of the relevant competitors. The conclusion from  
35 this is that the avidity of the fusion protein for the CEA epitope is comparable with that of the humanized



MAb 431/26. Furthermore, the fusion protein and the humanized MAb bind to the same epitope or to an epitope which lies spatially very near to that defined by the murine MAb BW 431/26 on CEA.

5      Example (I):

Demonstration of the tissue specificity of the 431/26<sub>hum</sub> V<sub>h</sub>hu $\beta$ Glc 1H fusion protein

10      Example (J) showed, inter alia, that the 431/26<sub>hum</sub> V<sub>h</sub>hu $\beta$ Glc 1H fusion protein is able to bind to purified CEA.

15      Example (K) showed that the V region of the fusion protein is able to compete with the V region of murine BW 431/26 for the same, or a very close, epitope. The indirect immunohistochemical assay which is specific for  $\beta$ -glucuronidase and is described hereinafter can be used to determine the microspecificity of the fusion protein on cryopreserved tissues.

The assay is described below:

- 20      - 5  $\mu$ m-thick frozen sections are placed on slides and dried in air for at least 30 minutes.
- The slides are subsequently fixed in acetone at -20°C for 10 seconds.
- The slides are washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 25      - 20-100  $\mu$ l of fusion protein-containing BHK cell supernatant (concentrated or diluted in tris/BSA, pH 7.4) is applied to each section and incubated in a humidity chamber at R.T. for 30 minutes.
- The slides are washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 30      - 50  $\mu$ l of hybridoma supernatant of the murine anti- $\beta$ -glucuronidase MAb BW 2118/157 are added to each section, and the slides are incubated in a humidity

chamber at R.T. for 30 minutes.

- The slides are then washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 5 - 20-100  $\mu$ l of bridge Ab (rabbit-antimouse IgG diluted 1:100 in human serum, pH 7.4) are applied to each section and incubated in a humidity chamber at R.T. for 30 minutes.
- The slides are then washed in tris/NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 10 - Subsequently 20-100  $\mu$ l of APAAP complex (mouse anti-AP diluted 1:100 in tris/BSA, pH 7.4) are applied to each section and incubated in a humidity chamber at R.T. for 30 minutes.
- The slides are then washed in tris-NaCl washing buffer, pH 7.4, with 0.1% BSA for 5 minutes.
- 15 - The substrate for alkaline phosphatase is made up as follows (100 ml of substrate solution sufficient for one glass cuvette):  
Solution 1: 3.7 g of NaCl  
20 2.7 g of tris base (dissolve in 75 ml of distilled water)  
+ 26.8 ml of propanediol buffer, pH 9.75, adjust with HCl  
+ 42.9 mg of levamisole = clear, colorless solution
- 25 Solution 2: Dissolve 21.4 mg of sodium nitrite in 535  $\mu$ l of distilled water = clear, colorless solution
- 30 Solution 3: Dissolve 53.5 mg of naphthol AS BI phosphate in 642  $\mu$ l of dimethylformamide (DMF) = clear, yellowish solution
- Add 368  $\mu$ l of 5% strength new fuchsin solution to solution 2 (sodium nitrite) and leave to react for 1 minute (stopclock) to give a clear, brown solution
- 35 - Add solution 2 (sodium nitrite with new fuchsin) and solution 3 (naphthol AS BI phosphate) to solution 1 (tris/NaCl/propanediol buffer) = clear,

yellowish solution

- adjust to pH 8.8 with HCl - cloudy, yellowish solution
- filter solution and place on the slide and leave to react on a shaker for 15 minutes - solution becomes cloudy.
- wash slide in tris/NaCl buffer, pH 7.4, for 10 minutes
- wash slide in distilled water for 10 minutes
- after drying in air for 2 hours, the slides are sealed in Kaiser's glycerol/gelatin at 56°C.

Specific binding of the fusion protein was demonstrated under the light microscope by the red coloration of the epitope-positive tissue sections. Comparative investigations with the murine MAb BW 431/26, which was detected by the indirect APAAP technique (Cordell et al., J. Histochem. Cytochem. 32, 219, 1984), revealed that the tissue specificity of the fusion protein agreed exactly with that of the murine MAb BW 431/26, i.e. that there is identity both of the reaction type in the individual specimen and of the number of positive and negative findings from a large number of different carcinomas and normal tissue.

#### Example (M):

#### Purification of the 431/26hum V<sub>H</sub>hu $\beta$ Glc 1H fusion protein

Murine and humanized MAbs can be purified by immunoaffinity chromatography methods which are selective for the Fc part of these molecules. Since there is no Fc part in the 431/26hum V<sub>H</sub>hu $\beta$ Glc 1H fusion protein, it was necessary to develop an alternative highly selective immunoaffinity chromatography method. Besides the selectivity of this method to be developed, it is necessary for the isolation conditions to be very mild in order not to damage the  $\beta$ -glucuronidase, which is very labile in the acidic and in the alkaline range.

5 The principle of the method comprises purification of the  
fusion protein from supernatants from transfected BHK  
cells using an anti-idiotypic MAb directed against the  
humanized V region. The preparation of such MABs is known  
from the literature (Walter et al., Behring Inst. Mitt.,  
82, 182-192, 1988). This anti-idiotypic MAB can be both  
murine and humanized. The MAB is preferably immobilized  
on a solid phase so that its V region has not been  
damaged. Examples of this are known from the literature  
10 (Fleminger et al., Applied Biochem. Biotechnol., 23, 123-  
137, 1990; Horsfall et al., JIM 104, 43-49, 1987).

15 The anti-idiotypic MAB thus immobilized on the solid phase  
by known methods binds very efficiently the fusion  
protein to be purified from transfected BHK cells, for  
example at pH 7, but has the surprising property that it  
no longer binds the fusion protein when the pH is lowered  
by only 1.5, to pH 5.5. This mild pH elution technique  
has no adverse effect on the fusion protein, either in  
its ability to bind to CEA or in its enzymatic activity  
20 (for methods, see Example J). Tab. 5 shows the OD values  
and fluorogenic units (FU) of the individual fractions  
from a purification using the solid phase-immobilized,  
anti-idiotypic MAB BW 2064/34.

Table 5:

Anti-idiotypic affinity chromatography

	Fractions	OD in %	FU in %	pH	Chromatography procedure
5	1-5	1	0	7.2	Preliminary washing of the column with PBS, pH 7.2
	6-142	20	0	7.2	Sample loading
	143-162	1	0	7.2	Washing of the column with PBS, pH 7.2
10	163	1	0	7.2	
	164	1	0	7.2	
	165	1	0	7.2	
	166	1	0	6.8	
15	167	2	10	6.1	
	168	5	20	5.7	
	169	16	40	5.6	
	170	23	80	5.5	
	171	26	100	5.4	Elution with PBS, pH 4.2
20	172	24	80	5.3	
	173	19	60	5.2	
	174	14	40	5.2	
	175	10	30	5.1	
	176	8	25	5.1	
25	177	6	20	5.1	
	178	3	10	5.0	
	179	2	5	5.0	
	180	1	0	5.0	

1 fraction = collection for 6.6 min (at pumping rate of 18 ml/h)  
 = 2 ml  
 The FU values are indicated as % of the highest value (fraction 171).

The elution of the fusion protein was measured as protein by measurement of the OD at 280 nm. In addition, the isolated fractions were examined for specific binding to CEA and simultaneous enzyme activity in the specificity/ enzyme activity assay (Example J). The values show that all the specific binding and enzyme activity was concentrated in one peak (peak eluted from around pH 5.0 to pH 5.6). The conclusion from this is that the described method of anti-idiotypic affinity chromatography is a very efficient and selective purification technique for the 431/26hum V<sub>H</sub>hu $\beta$ Glc 1H fusion protein.

Example (N):

Gel chromatography of the fusion proteins

The supernatants from the BHK cells secreting the 431/26hum V<sub>H</sub> hu $\beta$ Glc 1H fusion protein (B 70/6, B 74/2, B 72/72, B 73/2) were removed, sterilized by filtration and subjected to analytical gel filtration. For this, a TSK G3000 SW-XL column (7.8 x 300 mm) was equilibrated with 0.1 M sodium phosphate buffer, pH 6.7, + 0.02% NaN<sub>3</sub>, 20  $\mu$ l of the supernatant were loaded on, and elution was carried out with a flow rate of 0.6 ml/min. Starting with an elution time of 9 min (exclusion volume 9.5 min), fractions (0.3 min each) were collected and assayed for  $\beta$ -glucuronidase activity.

For this 25  $\mu$ l of the particular fraction were mixed with 75  $\mu$ l of substrate solution (2.5 mM 4-methylumbelliferyl  $\beta$ -glucuronide in 200 mM sodium acetate buffer, pH 5, + 0.1 mg/ml BSA) and incubated at 37°C for 2 hours. The reaction was then stopped with 1.5 ml of 0.2 M glycine/0.2% SDS solution, pH 11.7, and the fluorescent label liberated by the glucuronidase was quantified in a Hitachi fluorometer (with excitation wavelength of 360 nm and emission wavelength of 450 nm).

Result:

5 All 4 constructs show a single main activity peak between fractions 4 and 6 (Table 6). This corresponds to retention times of about 10.2 - 10.8 min. The fusion proteins with glucuronidase activity in the supernatants thus have retention times which are of the same order of magnitude as those of chemically prepared antibody- $\beta$ -glucuronidase constructs (10.4 min). The retention time for the free enzyme is 11.9, and for the free antibody is 12.3 min.

Table 6:

Gel filtration of various fusion proteins

Incubation: 25  $\mu$ l, 37°C, 120 min

Substance concentration: 1.875 mM; 0.1 mg/ml BSA

5 Each fraction 0.3 min; start at 9 min

		Liberated label/assay (FU)			
		B70/6	B74/2	B72/12	B73/2
10	1	11	17	15	15
	2	22	35	44	38
	3	125	97	873	1014
	4	1072	196	2959	3994
	5	1588	165	2206	3141
	6	1532	120	1133	1760
15	7	941	103	581	926
	8	710	69	376	723
	9	500	108	302	626
	10	371	123	316	613
	11	320	107	263	472
	12	254	91	210	456
20	13	224	57	146	357
	14	190	65	134	332
	15	171	52	83	294
	16	167	44	99	243
	17	100	39	73	217
	18	129	34	55	179
25	19	75	45	48	155
	20	66	41	36	129
	21				118
	22				93
	23				78
	24				61
30	25				24
	26				51



Example (O):

Molecular characterization of the 431/26 hum V<sub>H</sub> hu $\beta$ Glc1H fusion protein

5 The fusion proteins were purified by anti-idiotypa  
affinity chromatography in Example (M). Aliquots from the  
peak eluted at pH 5.5 were subjected to 10% SDS PAGE  
electrophoresis under non-reducing and reducing condi-  
tions and immunostained in a Western blot using anti-  
10 idiotypa MABs or with anti- $\beta$ -glucuronidase MABs (Towbin  
and Gordon (1979), Proc. Natl. Acad. Sci. USA 76: 4350-  
4354).

Under non-reducing conditions with the 431/26 hum V<sub>H</sub>  
hu $\beta$ Glc1H fusion protein, a main band of  $\approx$  125 kDa and a  
band of 250 kDa were detected and were detectable both by  
15 anti-idiotypa MAB and by anti- $\beta$ -glucuronidase MAB in the  
Western blot. Under reducing conditions there was no  
detectable immunostaining either by the anti-idiotypa or  
by the anti- $\beta$ -glucuronidase MABs. A 100 kDa and a 25 kDa  
band were detected in the reducing SDS PAGE. However,  
20 these molecules analyzed under denaturing conditions are,  
according to TSK G 3000 SW-XL gel filtration under native  
conditions in the form of a higher molecular weight  
product which has a molecular weight of  $\approx$  250 kDa  
(Example N). Diagrammatic representations of the 431/26  
25 hum V<sub>H</sub> hu $\beta$ Glc1H fusion protein are shown in Fig. M. Figure  
Mb shows the monomer which has a  $\approx$  25 kDa light chain and  
a  $\approx$  100 kDa heavy chain. This monomer and a dimer linked  
by inter-heavy chain disulfide bridges can be detected  
under denaturing conditions (Fig. Ma). Under native  
30 conditions, the fusion protein is in the form of a dimer  
of  $\approx$  250 kDa, with or without inter-heavy chain disulfide  
bridges (Fig. Mc).

## EXAMPLE (P):

## Chemical modification of the fusion protein

The fusion protein purified as in Example (N) (110  $\mu$ g/ml) was adjusted to pH 4.5 and mixed with sodium periodate (final concentration 1 mM). After incubation at room temperature in the dark for 1 hour, the sodium periodate was removed by gel chromatography, and the fusion protein was then readjusted to pH 8. Addition of ethanolamine to a final concentration of 0.1 M was followed by incubation at 4°C for a further 3 hours, then sodium cyanoborohydride (final concentration 5 mM) was added and incubated for 30 min (reduction). This was followed by another gel filtration to remove the reducing agent and to change the buffer of the fusion protein. The chemical modification had no effect on the functional activity of the fusion protein. Tab. 7 shows the change in the plasma concentrations of unmodified and modified fusion protein in the nude mouse. The elimination of the fusion protein from the plasma is greatly slowed down by the modification.

Table 7:

Plasma levels of  $\beta$ -glucuronidase activity in the nude mouse

25	t [min]	$\beta$ -Glucuronidase fusion protein	
		Treated % activity	Untreated % activity
	0	100	100
	10		54
30	30	76	
	60		22
	240	40	4
	480		1
	1380	19	
	1440		1
35	5880	3	

2062047

EXAMPLE (Q):

Enzymatic treatment of the fusion protein

53  $\mu$ g of fusion protein (Example N) in 0.01 M tris/HCl, 0.15 M NaCl were incubated with 1 unit of soluble alkaline phosphatase (E. coli) or immobilized alkaline phosphatase (bovine intestine) at room temperature for 20 h. Tab. 8 shows the change in the plasma concentration of untreated and treated fusion protein in the nude mouse. The elimination is not significantly affected by the enzyme treatment.

Table 8:

Plasma levels of  $\beta$ -glucuronidase activity in the nude mouse

t	$\beta$ -Glucuronidase fusion protein		
		AP (bovine intestine, immobil.)	AP (E. coli)
[min]	untreated [%]	treated [%]	treated [%]
0	100	100	100
10	78	76	65
30	44	57	53
60	35	36	35
240	22	27	22
1440	4	5	5

AP = alkaline phosphatase

Example (R):

Pharmacokinetics and tumor retention of the 431/26 hum V<sub>1</sub> hu $\beta$ Glc 1H fusion protein

By way of example, 5 x 4  $\mu$ g of purified fusion protein

which was mixed with 100  $\mu$ g of HSA/ml were injected in unmodified (Example N) and chemically modified form (Example P) i.v. at 24-hour intervals into CEA-expressing nude mice harboring human tumors. After defined time intervals, 3 animals in each case were sacrificed by cervical dislocation. The organs were removed, weighed and mixed with 2 ml of 1% strength BSA in PBS, pH 7.2. The tissue and cells from these organs were then broken down in a Potter (10 strokes) and the amount of functionally active fusion protein was determined in the supernatant after centrifugation of the suspension at 3000 rpm and RT for 10' (Heraeus Labofuge GL, Type 2202) in the specificity/enzyme activity assay (see Example J). The data from a representative experiment are shown in Tab. 9. It is clearly evident that the chemically modified fusion protein, which has a  $t_{1/2\beta}$  of  $\approx 4$  h, specifically accumulates in the tumor from  $\geq 3$  days after completion of the repetitive injection phase. The unmodified fusion protein, which has a  $t_{1/2\beta}$  of  $\approx 20$  min, showed no significant accumulation in the tumor under the same experimental conditions.

It may be concluded from these data that the hu 431  $\beta$ -Gluc fusion protein is able to bind in vivo to CEA-positive tumors and to remain there as enzymatically active molecule over long time periods ( $> 9$  days). The time the prodrug is administered in this system should be between day 3 and 9 after completion of the fusion protein injection.

Table 9:

Tumor retention experiment with CEA-positive human stomach carcinoma xenograft (Mz Sto 1)

i.v. Injection of  $5 \times 4 \mu\text{g}$  of fusion protein per mouse at intervals of 24 hours

Time (h) after last i.v. injection	% Activity/g of tumor or organ						
	Tumor	Liver	Lung	Spleen	Kidney	Intestine	Plasma
3	100	100	100	100	100	100	100
24	86.7	49.3	26.3	-	64	123	44.4
72	26.4	11	4.4	-	8.1	19	6.5
216	24.4	1.5	0.13	1.2	0.4	0.5	0.015

Example (S):

Isoelectric focusing of the 431/26 hum V<sub>H</sub> hu $\beta$ Glc 1H fusion protein

5 The fusion protein purified by anti-idiotypic affinity chromatography (Example N) was subjected to isoelectric focusing in the Pharmacia Fast System by the method of Righetti et al. (1979). It emerged from this that the isoelectric point of the molecule lies in a pH range from 7.35 to 8.15.

10 Example (T):

Demonstration that a cytostatic prodrug can be cleaved by the 431/26 hum V<sub>H</sub> hu $\beta$ Glc 1H fusion protein

15 It was shown in Example (J) that the  $\beta$ -glucuronidase portion of the fusion protein is able, like the native human enzyme, to cleave the  $\beta$ -glucuronide of 4-methylumbelliferol. In the investigations which are described hereinafter, the substrate used for the enzymatic cleavage was a  $\beta$ -glucuronide, linked via a spacer group, of the cytostatic daunomycin. The specific procedure for these investigations was as follows:

20 4 mg of the compound N-(4-hydroxy-3-nitrobenzyloxy-carbonyl)daunorubicin  $\beta$ -D-glucuronide (prodrug), which is described in French Patent Application (No. d'Enregistrement National: 9105326) were dissolved in 1 ml of 20 mM phosphate buffer, pH 7.2. 35  $\mu$ l of the fusion protein (Example N) or of human  $\beta$ -glucuronidase (total concentration in each case 6.5 U/ml; 1 U = cleavage of 1  $\mu$ mol of 4-methylumbelliferol/min at 37°C) were pipetted into 5  $\mu$ l portions of this substrate solution and incubated in the dark at 30 37°C. Samples (5  $\mu$ l) of the incubation mixture were removed after various times and immediately analyzed by high pressure liquid chromatography under the following conditions:

Column:

Nucleosil 10C RP 18, 5  $\mu$ m particle diameter, 125 x 4.6 mm

Mobile phase:

Gradient of solution A (100% acetonitrile) and solution B  
(20 mM phosphate buffer pH 3.0)

5

0 min: 30% solution A

15 min: 70% solution A

20 min: 70% solution A

Flow rate: 1 ml/min

10

Detection: fluorescence, excitation 495 nm, emission 560 nm

Data analysis: Beckman System Gold Software

15 The retention time of the starting compound (prodrug) under these chromatography conditions was 11 min. The compound produced during the incubation (drug) had a retention time of 8.9 min, identical to daunomycin (DNM, analysis of a standard under the same conditions). The kinetics of the cleavage of the starting compound by the fusion protein and human  $\beta$ -glucuronidase are shown in Tab. 11 and Tab. 10 respectively.

20 The half-life of the cleavage of the prodrug by the fusion protein was 2.3 h. Cleavage by human  $\beta$ -glucuronidase took place with a half-life of 0.8 h. As already demonstrated in Example (J), the results of the investigations show that the  $\beta$ -glucuronidase portion of the fusion protein is  
25 functionally active and able to cleave  $\beta$ -glucuronides. The kinetics of the elimination of the glucuronide portion and the liberation of the drug (daunomycin) from the prodrug used show a rate comparable in magnitude to human  
30  $\beta$ -glucuronidase, so that the substrate specificity of the fusion protein essentially agrees with that of human  $\beta$ -glucuronidase.

Table 10:

Kinetics of prodrug cleavage by  $\beta$ -glucuronidase (human, recombinant)

	t min	Prodrug area %	DNM area %
5	0	99.2	0.8
	57	36.0	64.0
	130	10.3	89.7
	227	9.3	90.7
10			

Table 11:

Kinetics of prodrug cleavage by  $\beta$ -glucuronidase (fusion protein)

	t min	Prodrug area %	DNM area %
15	0	98.9	1.1
	50	81.1	18.9
	135	51.7	48.3
	190	33.0	67.0
20	247	22.0	78.0
	317	12.4	87.6



THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE  
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A fusion protein for prodrug activation of the formula  
huTuMAB-L- $\beta$ -Gluc, where huTuMAB is a humanized tumor-  
specific monoclonal antibody or a tumor-binding fragment  
5 thereof, L is a linker, and  $\beta$ -Gluc is human  
 $\beta$ -glucuronidase.
2. A fusion protein as claimed in claim 1, in which the  
antibody fragment is composed of a V<sub>H</sub> exon, a CH<sub>1</sub> exon  
and a hinge exon and leads to expression of fusion  
10 proteins which are not linked together by disulfide  
bridges.
3. A fusion protein as claimed in claim 1, in which the  
antibody fragment is composed of a V<sub>H</sub> exon, a CH<sub>1</sub> exon  
and two hinge exons and leads to the expression of  
15 fusion proteins in which the heavy chains fragments  
are linked together by disulfide bridges.
4. A fusion protein as claimed in claim 1, in which the  
antibody fragment is composed of a V<sub>H</sub> exon, a CH<sub>1</sub> exon  
and three hinge exons and leads to the expression  
20 of fusion proteins which are linked together by  
disulfide bridges.
5. A fusion protein as claimed in claim 1, in which the  
antibody fragment is composed of a V<sub>H</sub> exon and a CH<sub>1</sub>  
exon and on expression can associate with a modified  
25 light chain composed of V<sub>L</sub> and CH<sub>1</sub> domain.
6. A fusion protein as claimed in any of claims 1 to 5,  
where the huTuMAB portion derives from MAb BW 431/36.
7. A fusion protein as claimed in any of claims 1 to 5,  
where the huTuMAB portion is formed from one of the  
30 MAb described in EP-A1-0 388 914.
8. A fusion protein as claimed in any of claims 1 to 7,

in which L contains a polypeptide spacer as shown in Tab. 1 and/or 2.

9. A fusion protein as claimed in any of claims 1 to 8,  
in which L contains 1, 2 or 3 hinge regions of a human  
IgG3 C gene.
10. A plasmid which contains the cDNA for peptides as  
claimed in claim 1 to claim 9.
11. A transformed eukaryotic cell which is transformed  
with a plasmid as claimed in claim 10.
12. A process for the preparation of proteins as claimed  
in claim 1 to claim 9, which comprises expressing these  
fusion proteins in transformed cells by means of  
plasmids, and isolating said fusion proteins via anti-  
idiotype MAbs.
13. A fusion protein as claimed in any of claims 1 to 9,  
which fusion protein is treated with an oxidizing agent.
14. A fusion protein as claimed in claim 13, which fusion  
protein is reductively aminated in a second reaction  
step.
15. A process for the preparation of fusion proteins as  
claimed in claim 13, which comprises treating the fusion  
proteins as claimed in any of claims 1 to 9 with an  
oxidizing agent.
16. A process for the preparation of fusion proteins as  
claimed in claim 14, which comprises the fusion proteins  
as claimed in any of claims 1 to 9 being oxidized in  
a first reaction step and reductively aminated in a  
second reaction step.
17. A fusion protein as claimed in any of claims 1 to 9  
and claims 13 and 14 as pharmaceutical.

18. Information provided as set forth in any of claims 1 to 9 and claims 13 and 14 as diagnostic aid.

2062047

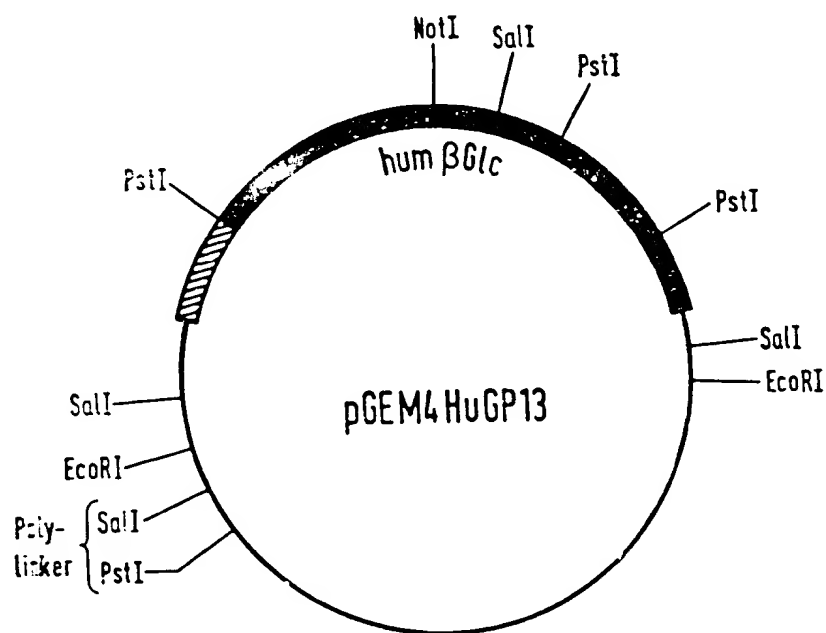


Fig. A

By: Bereskin & Parr

2062047

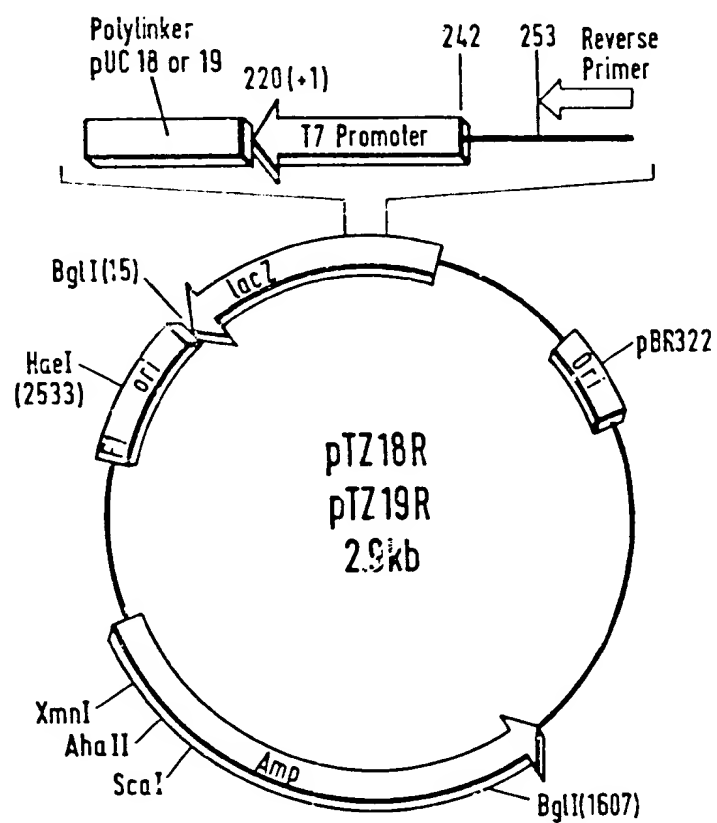


Fig. B.1

By: Bereskin & Parr

2062047

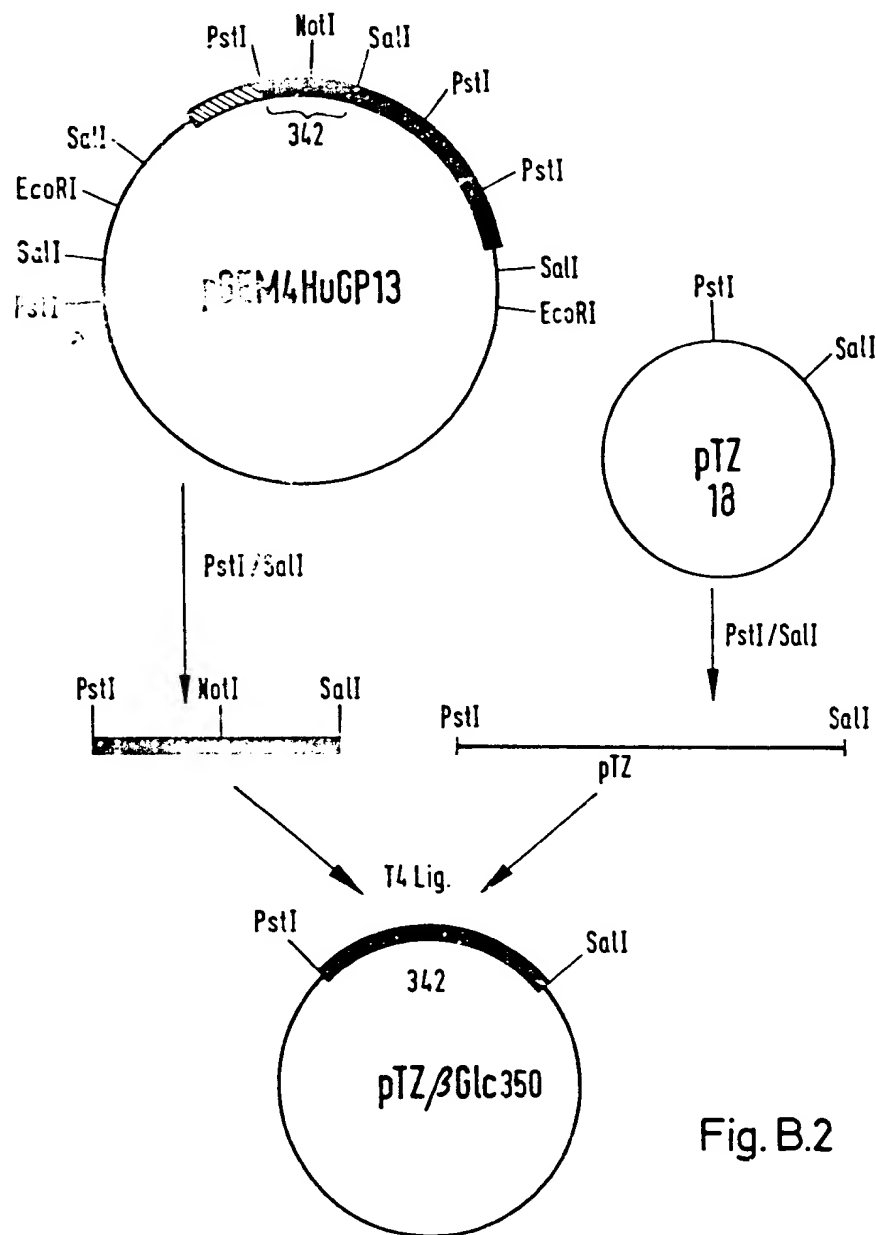
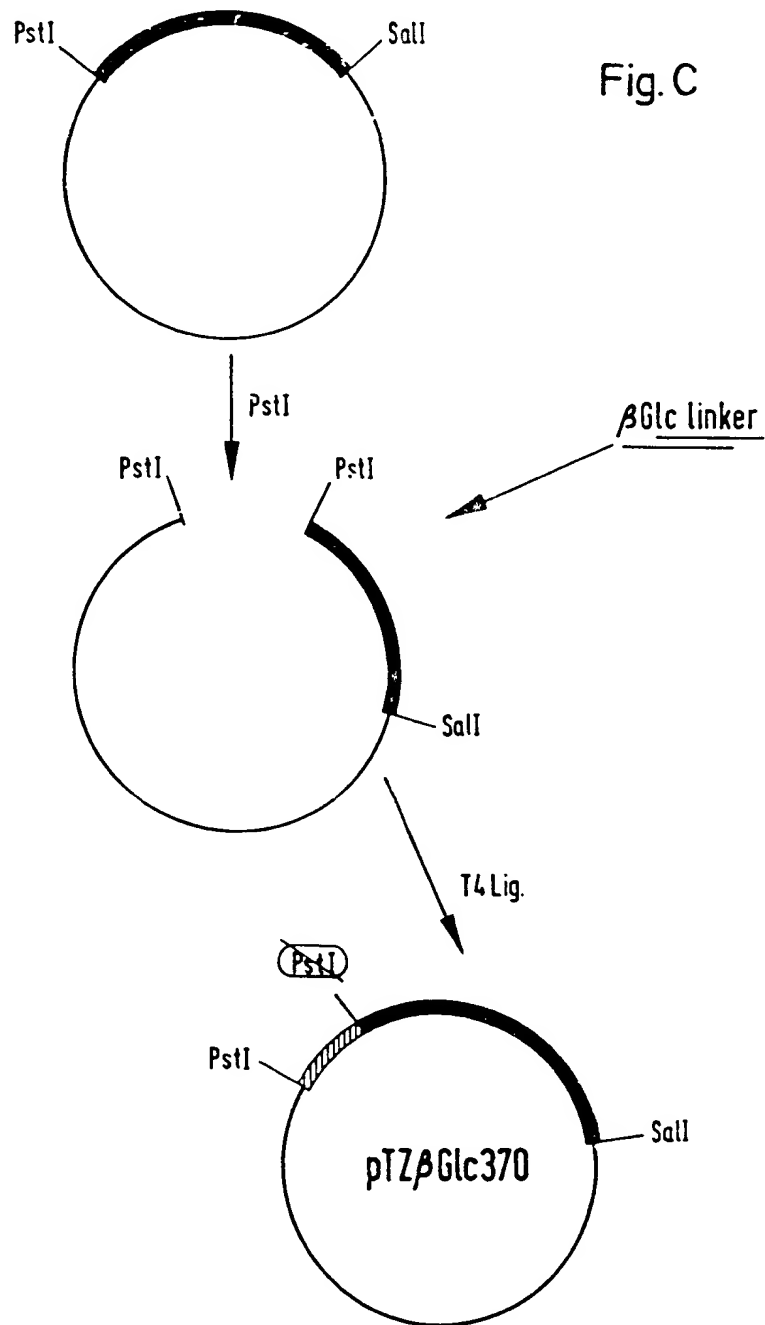


Fig. B.2

by: Bereskin & Parr

2062047

Fig. C



By. Beresford & Parr

2062047

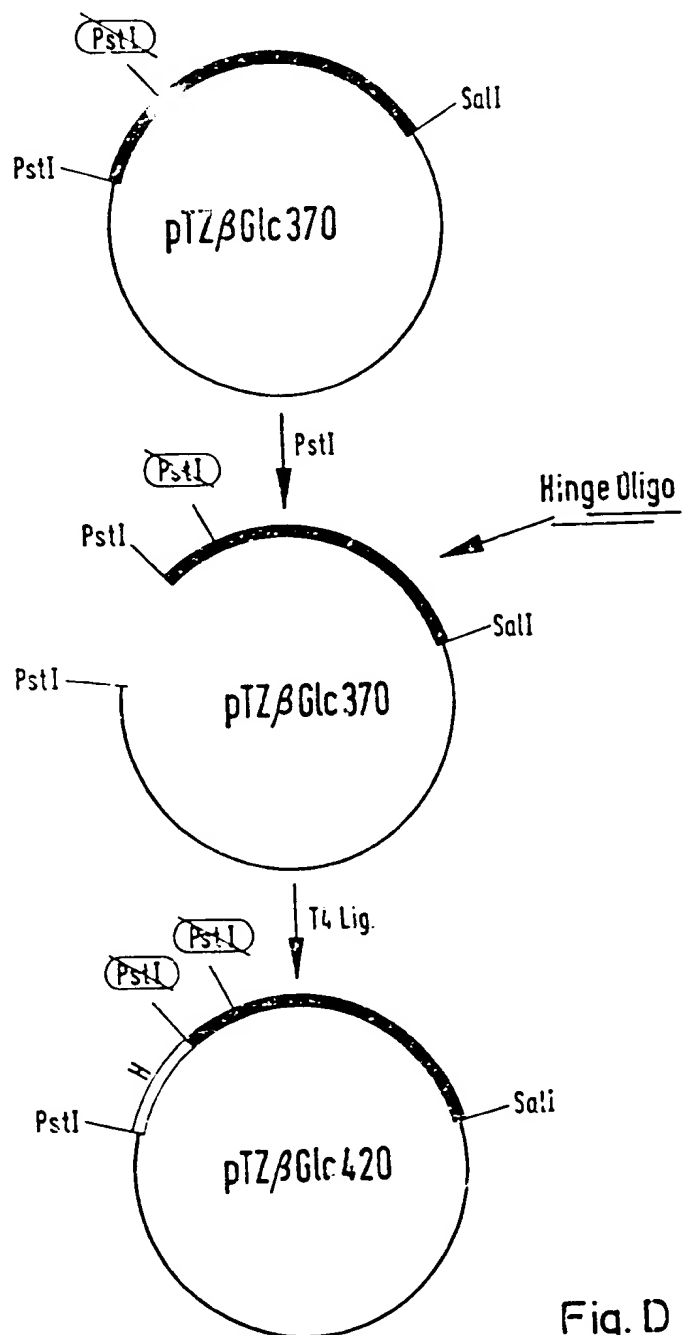


Fig. D

By: Bessie & Pan



2062047

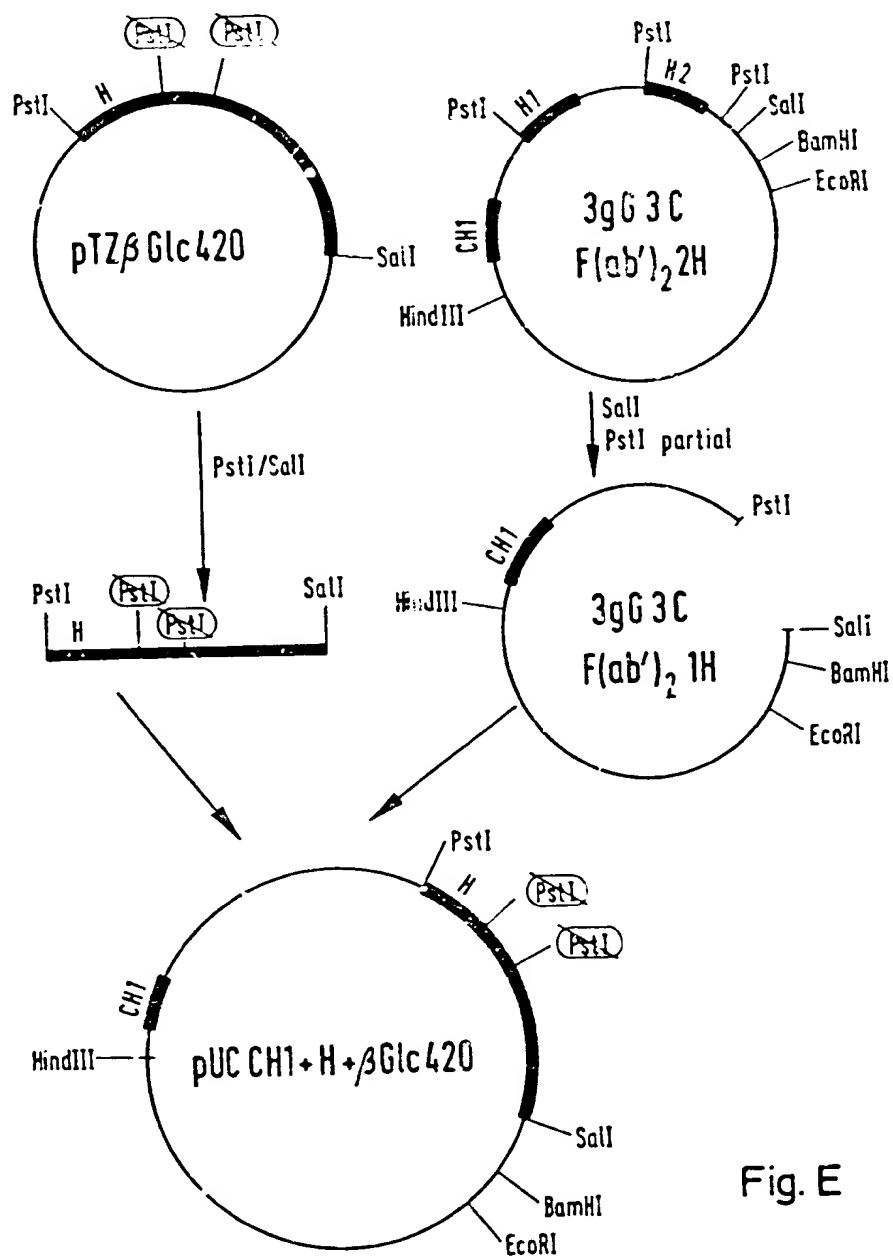


Fig. E

By: Blumberg & Parr

2062047

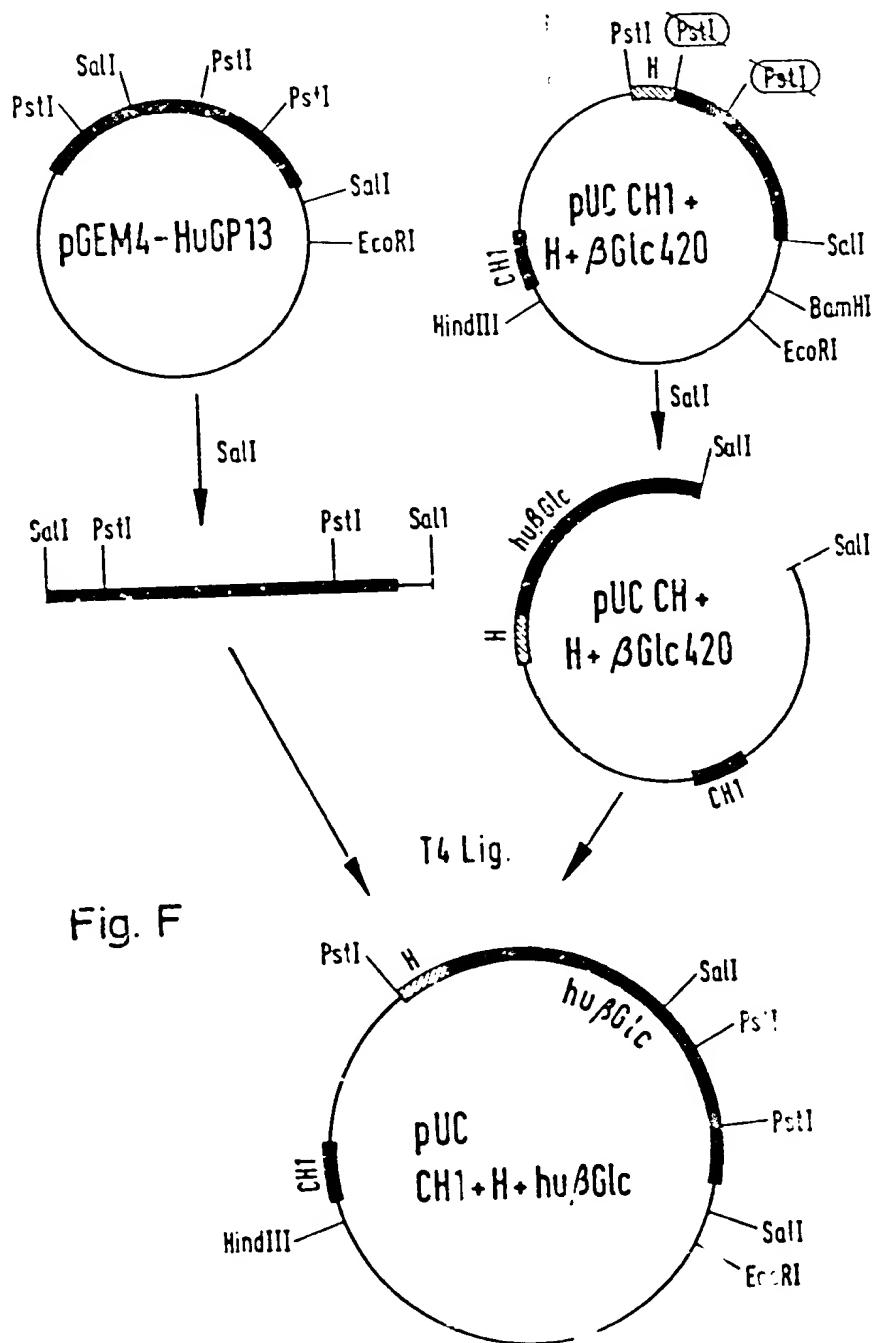


Fig. F

By: Keresztes & Pan

2062047

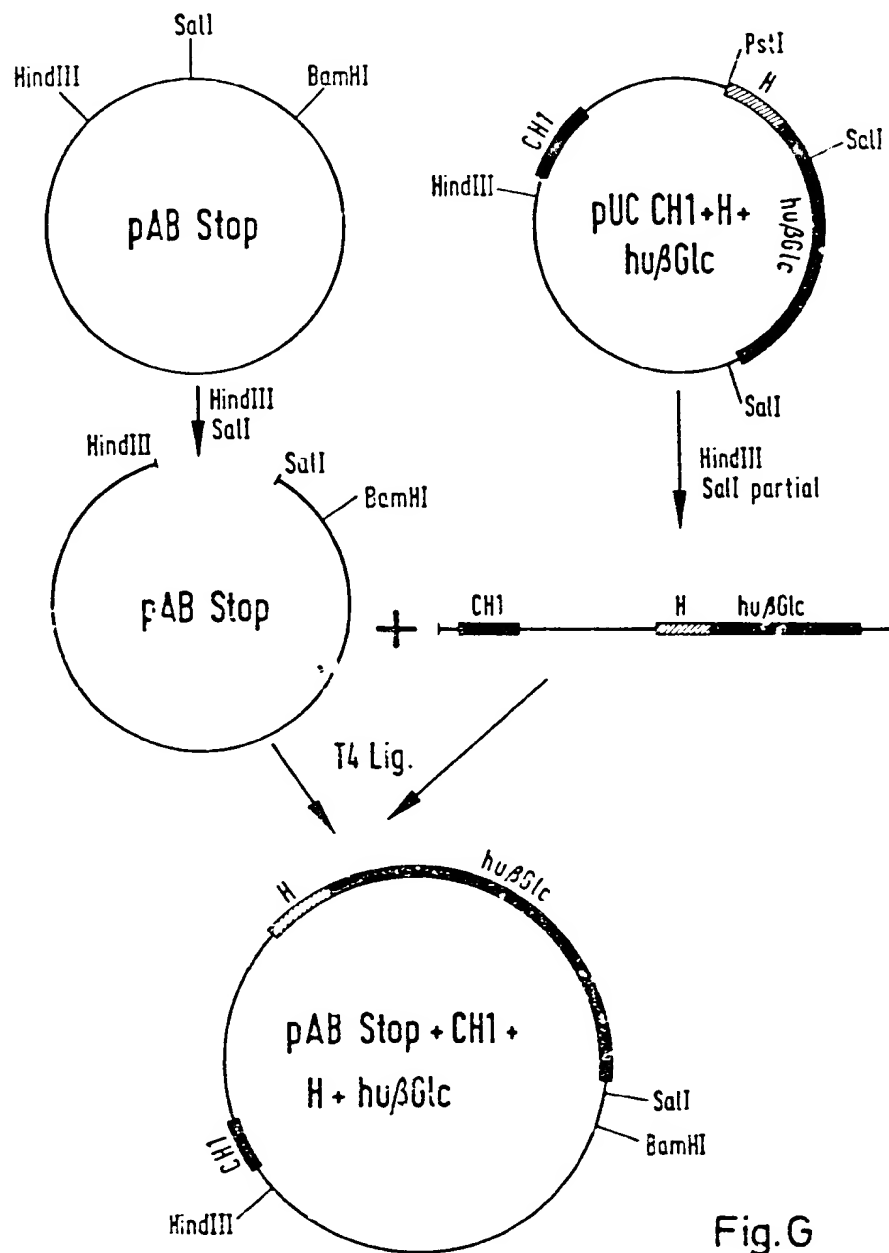


Fig.G

Fig. Blecker & Parn

2062047

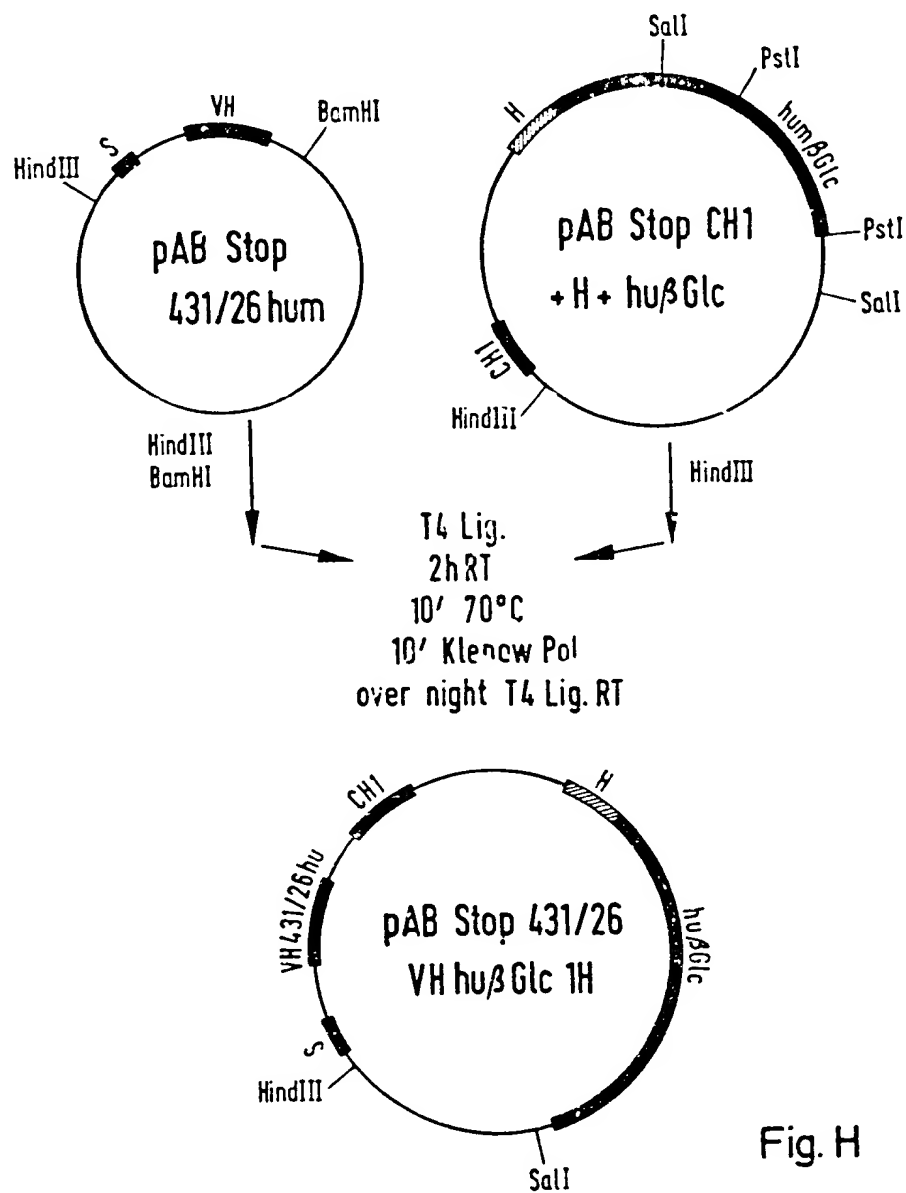


Fig. H

By: Bernstein & Parr

2062047

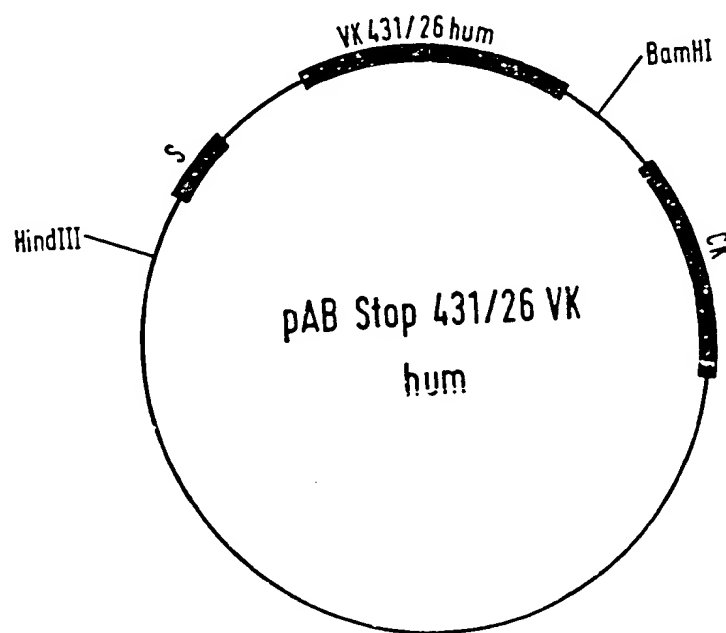


Fig. I

By. Bereskin & Parr

2062047

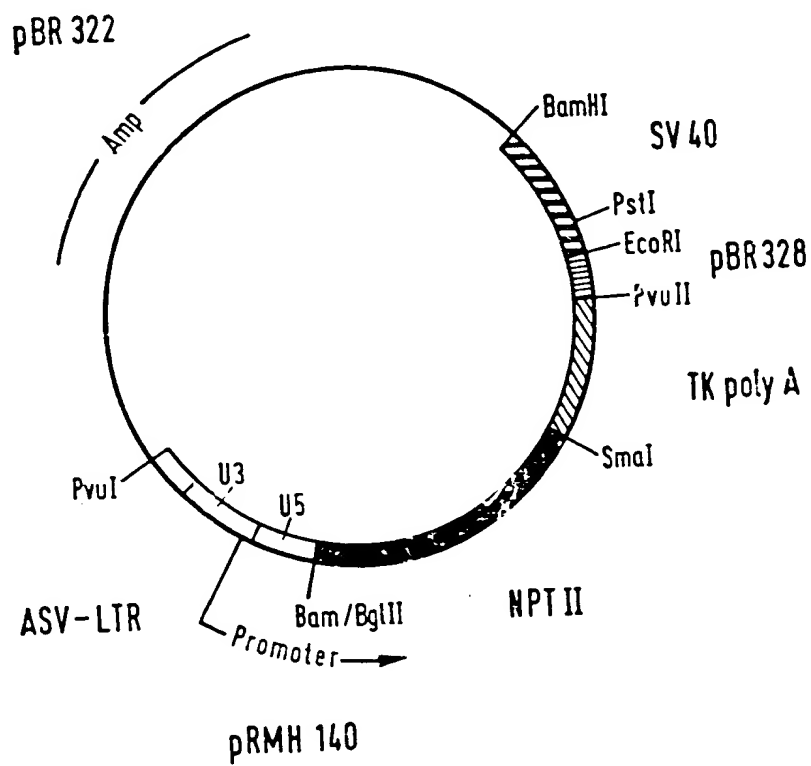


Fig. K

By: Bereckin + Pan

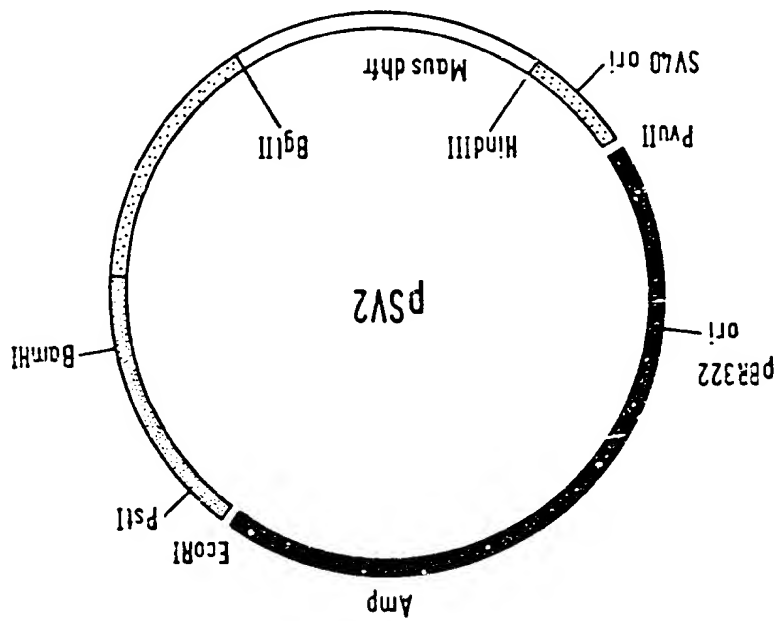
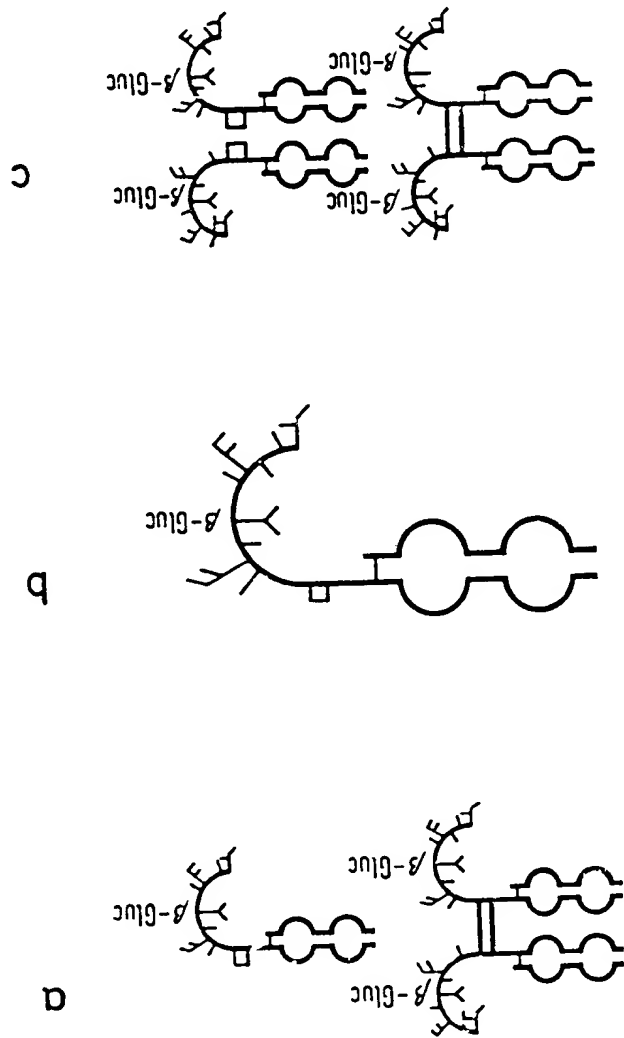


Fig. 1

by: Beretta, & Pan

Fig. M



by: Bessie + Pan